

## SPECIFICATION

### FOR

## A COMPOUND AND METHOD FOR THE TREATMENT OF SINUSITIS

### BACKGROUND OF THE INVENTION

5 [0001] This invention relates to the field of pathologies of the mucosal membranes of the head and facial sinus cavities caused by inflammation.

#### Introduction

[0002] The sinuses are air pockets located inside the bones in the skull. They are located to either side of the nose (maxillary), behind and in between the eyes (ethmoid), in the forehead (frontal), and there is one much further back in the head (sphenoid). Each sinus is drained by a small hole, about 4mm in diameter, called an ostium. The sinuses are lined with very fine hair-like projections called cilia. The function of the cilia is to encourage the drainage of mucus.

[0003] Sinusitis is frequently caused by an obstructed ostium. This obstruction may result from an anatomical defect such as a deviated septum, inflammation due to an upper  
15 respiratory infection or an allergic response, drying of the mucus, or a foreign body caused from an accident. When this occurs, mucus that normally is expelled from the sinus builds up in the sinus causing pain, pressure and an excellent culture medium for bacteria. If the mucus is not cleared immediately, an abscess can develop in the sinus. Unfortunately, draining the abscess is not feasible without doing extensive surgery.

### **Bacterial Sinusitis**

[0004] Sinusitis is one of the most common medical problems affecting approximately 30% of the population at some point. The most common pathogens associated with acute sinusitis are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*.

5 [0005] Many strains of both *H. influenzae* and *M. catarrhalis* now are beta-lactamase-producing and therefore not susceptible to beta-lactamase antibiotics. Accordingly, broad spectrum antibiotics such as amoxicillin/clavulanate potassium cefuroxime axetil, cefpodoxime proxetil, clarithromycin, and azithromycin are often employed.

10 [0006] The use of decongestants in sinusitis is somewhat controversial. Some authorities feel that these agents can help to remove the offending matter from the sinus cavities, permitting greater effectiveness for antimicrobial treatment. Others, however, believe that decongestants have no proven role in acute sinusitis and should be avoided because they have side effects and add to the overall cost of treatment. These experts also feel that complicating the therapeutic regimen by having the patient take a decongestant is likely to decrease the likelihood of compliance with the antibiotic treatment.

[0007] Chronic sinusitis is defined as sinusitis persisting for at least three weeks. Typically, early in the development of a sinus infection, the cilia are lost and the mucus becomes increasingly thick.

15 [0008] Whereas acute sinusitis is a bacterial infection usually relating to prior viral  
20 respiratory tract infection, persistent or chronic sinusitis appears to have a noninfectious

etymology. Antimicrobial treatment may clear the condition temporarily, but failure to address and successfully resolve the underlying problem ensures that sinusitis will recur.

[0009] A number of conditions can predispose a person to chronic or recurrent sinusitis, including principally, allergic rhinitis. The presentation of chronic sinusitis may be quite subtle, consisting only of congestion, cough, and postnasal drainage; a high index of suspicion is important in identifying possible predisposing factors. The nasal discharge associated with allergic rhinitis is typically clear and watery, and the patient may have symptoms that include itching of the nose and/or eyes.

[0010] Among the less common but nonetheless worrisome causes of persistent sinusitis are mechanical obstructions such as polyps or foreign bodies, or mucosal inflammation resulting from disorders such as immunoglobulin deficiencies, cystic fibrosis, and trauma.

[0011] Mild cases of chronic sinusitis are usually treated with a regimen of antihistamines/decongestants and/or corticosteroids. Antihistamines may include pseudoephedrine, phenylephrine, phenylpropanolamine, chlorpheniramine, brompheniramine, pheniramine and loratidine. Steroid nasal sprays are commonly used to reduce inflammation in chronic sinusitis. Although these nasal sprays are occasionally used for long-term treatment for patients with chronic sinusitis, the long-term safety of these medications, especially in children, is not fully understood, and the benefits and risks need to be balanced. For patients with severe chronic sinusitis, a doctor may prescribe oral steroids, such as prednisone. Other suitable corticosteroids include betamethasone, cortisone, dexamethasone, hydrocortisone, methylprednisone, and triamcinolone. Because oral steroids can have significant side effects, they are prescribed only when other medications have not been effective. Similar complications

exist for antihistamines. Since vascularization of the sinuses is poor, it is difficult to achieve pharmacologically effective concentrations of drugs. As a consequence sinusitis sufferers are often put on lengthy treatments at high dosage, often prescribed in dosages exceeding 2g per day, from at minimum 2 weeks often extending up to six or eight weeks.

- 5 [0012] When drug therapies fail to treat the sinusitis, irrigation of saline or saline in combination with antibiotics/antiinflammatories may be applied to treat sinusities of the sinuses immediately downstream from the relevant ostiums passage. If irrigation is ineffective, surgery is often necessary to manually clear the obstructed sinus passages.

### Fungal Sinusitis

[0013] Research from the Mayo clinic has suggested that sinusitis, especially, chronic sinusitis may have a fungal component to its causation.

[0014] Fungal growth was found in washings from the sinuses in 96% of patients with chronic sinusitis. Normal controls had almost as much growth, the difference being that those patients with chronic sinusitis had eosinophiles which had become activated. As a result of the activation, the eosinophiles released Major Basic Protein (MJP) into the mucus, which attacks and kills the fungus but may cause inflammation to the underlying epithelium.

[0015] Fungal sinusitis is broken down into several categories: Allergic, Fungus balls (Mycetoma), and Invasive.

- 20 [0016] Allergic fungal sinusitis (AFS) is commonly caused by Aspergillus, as well as Fusarium, Curvularia, and others. Patients often have associated asthma. The criteria include

CT or MRI confirmation, a dark green or black material the consistency of peanut butter called "allergic mucin" which typically contain a few hyphae, no invasion, and no predisposing systemic disease. Charcot-Leyden crystals, which are breakdown products of eosinophiles are often found. Usually patients are found to be allergic to the fungus, although this is controversial. This disease is analogous to Allergic Bronchopulmonary Aspergilosis.

[0017] Fungus balls often involve the maxillary sinus and may present similarly to other causes of sinusitis including helitosis. In addition to radiological abnormalities, thick pus or a clay-like substance is found in the sinuses. There is no allergic mucin, but dense non-innervated hyphae are found. There is an inflammatory response in the mucosa. Upon looking into the sinus, the fungus ball can vary in size from sub-millimeter to the dimensions of the sinus. The fungus balls may have a greenish-black appearance. Removal of the fungus ball is the typical treatment.

[0018] Invasive sinusitis can progress rapidly, and typically necessitates surgery, often on an emergency basis and often requiring the intravenous administration of Amphotericin B as well. There have been some forms of invasive sinusitis, which can cause proptosis.

[0019] Fungal based sinusitis may be treated with topical fungicides alone or in combination with broad-spectrum antibiotics and corticosteroids. Usually, the fungicide/antibiotic are mixed with a saline irrigation solution and administered as part of irrigation therapy.

[0020] Accordingly, it is an object of the present invention to provide a composition and a method of treatment of bacterial and fungal sinusitis while minimizing or eliminating some or all of the complications associated with current antibiotic, anti inflammatory and/or antifungal

therapies. It is another object of the present invention to provide a composition and method of treatment effective against chronic and acute sinusitis of either bacterial, fungal or allergic etymology.

### SUMMARY OF THE INVENTION

5 [0021] The invention includes a composition and method of treatment of sinusitis. A preferred embodiment of the invention is composition for the treatment of sinusitis comprising a therapeutically effective amount of one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV in combination with a therapeutically effective amount of an antihistamine/decongestant.

[0022] Another preferred embodiment of the invention is a composition for the treatment of sinusitis comprising a therapeutically effective amount of one or more of these peptides in combination with a therapeutically effective amount of a corticosteroid.

[0023] In another embodiment of the invention each of these compositions may comprise a therapeutically effective amount of an antibiotic.

15 [0024] In another embodiment of the invention each of these combination compositions may further comprise a therapeutically effective amount of a fungicide.

[0025] More preferably still, the peptides in each of these preferred combination compositions has the primary sequence of KPV or VPK-Ac-CC-Ac-KPV (Ac=Acetyl group). In all the preferred compositions, pharmacologically effective concentrations of the peptides may  
20 be as low as  $10^{-12}$ M but may be as high  $10^{-4}$  M.

[0026] In another embodiment of the invention a peptide of one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV is topically or systemically applied to treat sinusitis.

[0027] In another embodiment of the invention one or more of these preferred peptides used in combination with therapeutically effective amounts of antihistamines/decongestants, corticosteroids, antibiotics, and/or fungicides, are topically or systemically applied to treat sinusitis.

[0028] In yet another embodiment of the invention, one or one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV, which may or may not be in combination with therapeutically effective amounts of antibiotics, corticosteroids, fungicides and/or antihistamine/decongestants, are topically or systemically applied before, during or after surgery to treat sinusitis.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0029] Figure 1 illustrates the effect of  $\alpha$ -MSH (1-13) and (11-13) and the peptide VPK-Ac-CC-Ac-KPV on *C. albicans* colony forming units compared to controls. All three molecules significantly decreased *C. albicans* colony forming units over a broad range of peptide concentrations.

[0030] Figure 2 represents a comparison of candidacidal activity of certain melanocortin peptides and fluconazole (all  $10^{-6}$ M). The most effective of the melanocortin peptides were

those including the C-terminal amino acid sequence of  $\alpha$ -MSH, namely,  $\alpha$ -MSH (1-13), (6-13) and (11-13).

[0031] Figure 3A shows untreated germination of *C. albicans*, i.e. blastopores.

[0032] Figure 3B shows a horse serum-induced germination of *C. albicans*.

5 [0033] Figure 3C shows the effect of  $\alpha$ -MSH (1-13) treatment on germination of *C. albicans*

[0034] Figure 3D shows the effect of  $\alpha$ -MSH (11-13) treatment on germination of *C. albicans*

[0035] Figure 4 illustrates the effect of  $\alpha$ -MSH (1-13) and (11-13) on *C. albicans* killing by human neutrophils. Values are expressed as percent increase in killing vs. medium along. Scores are means  $\pm$  SEM.

[0036] Figure 5 illustrates the effect of  $\alpha$ -MSH (1-13), (11-13), and forskolin on cAMP content of *C. albicans*.

15 [0037] Figure 6 illustrates the inhibitory effect of  $\alpha$ -MSH (1-13), (11-13), and forskolin on *C. albicans* colony forming units.

[0038] Figure 7 illustrates the anti-inflammatory effects  $\alpha$ -MSH(11-13) relative to hydrocortisone on edema caused by injection of cargeenan . Scores are mean  $\pm$  SEM differences from baseline measures for control and peptide treatments.



[0039] Figure 8 illustrates inhibition by  $\alpha$ -MSH(11-13) of inflammation induced in the ear by picryl chloride application. Scores are mean  $\pm$  SEM differences from baseline measures for control and peptide treatments. Number of animals shown in parentheses.

[0040] Figure 9 illustrates inhibition of inflammation by Ac-[D-Lys<sup>11</sup>] $\alpha$ -MSH(11-13)-NH<sub>2</sub>. Scores are mean  $\pm$  SEM differences from baseline measures for control and peptide treatments. Number of animals shown in parentheses.

[0041] Figure 10 illustrates the lack of inflammation by Ac-[D-Pro<sup>12</sup>] $\alpha$ -MSH(11-13)-NH<sub>2</sub>. Scores are mean  $\pm$  SEM differences from baseline measures for control and peptide treatments. Number of animals shown in parentheses.

[0042] Figure 11 illustrates the inhibition of inflammation by Ac[D-Val<sup>13</sup>] $\alpha$ -MSH(11-13)-NH<sub>2</sub>. Scores are mean  $\pm$  SEM differences from baseline measures for control and peptide treatments. Number of animals shown in parentheses.

[0043] Figure 12 illustrates inhibition of inflammation by Ac-[D-Lys<sup>11</sup>, D-Val<sup>13</sup>] $\alpha$ -MSH(11-13)-NH<sub>2</sub>. Scores are mean  $\pm$  SEM differences from baseline measures for control and peptide treatments. Number of animals shown in parentheses.

[0044] Figure 13 illustrates the relative inhibition of  $\alpha$ -MSH(11-13)'s anti-inflammatory action on edema caused by intradermal injection of human recombinant 1L1 $\beta$  in the mouse ear. "IP" refers to intraperitoneal injection 1 "Sal" refers to a saline solution. "ICV" refers to an intracerebroventricular injection.

[0045] Figure 14 illustrates the relative inhibition of the anti-inflammatory actions of centrally administered  $\alpha$ -MSH(11-13) by  $\beta_2$ -adrenergic antagonist butoxamine. Atenolol is a  $\beta_1$ -adrenergic antagonist.

[0046] Figure 15 illustrates the relative inhibition of the anti-inflammatory actions of centrally administered  $\alpha$ -MSH(11-13) in the presence of an  $\alpha$ -receptor antagonist. Phentolamine is a cholinergic receptor antagonist.

[0047] Figure 16 illustrates the relative inhibition of the anti-inflammatory actions of centrally administered  $\alpha$ -MSH(11-13) as a function of time in spinal cord transected mice.

[0048] Figure 17 illustrates the relative inhibition of the anti-inflammatory actions of systemically administered  $\alpha$ -MSH(11-13) as a function time in spinal cord transected mice.

[0049] Figure 18 illustrates the relative inhibition of the anti-inflammatory actions of systemically administered  $\alpha$ -MSH(11-13) as a function time in spinal cord transected mice.

[0050] Figure 19 shows the relative change in concentrations of  $\text{NO}_2^-$ , IL-6 and  $\text{TNF-}\alpha$  as a function of  $\alpha$ -MSH(11-13) concentration in activated microglia.

15 [0051] Figure 20 shows the relative change in concentrations of  $\text{NO}_2^-$ , IL-6 and  $\text{TNF-}\alpha$  as a function of  $\alpha$ -MSH(1-13) concentration in activated microglia.

[0052] Figure 21 shows the relative change in concentrations of  $\text{NO}_2^-$ , IL-6 and  $\text{TNF-}\alpha$  as a function of ACTH (1-24) concentration in activated microglia.

[0053] Figure 22 shows the relative accumulation of cAMP as a function of  $\alpha$ -MSH(1-13) and  $\alpha$ -MSH(11-13) concentration in resting microglia.

[0054] Figure 23 shows the relative change in concentrations of  $\text{NO}_2^-$ , IL-6 and TNF- $\alpha$  as a function of  $\alpha$ -MSH(11-13) microglia co-incubated with LPS + IFN- $\alpha$ .

5 [0055] Figure 24 is a Northern Blot showing the inhibitory influences of melanocortin peptides (ACTH (1-24),  $\alpha$ -MSH(1-13),  $\alpha$ -MSH(11-13)) on TNF- $\alpha$  and  $\text{NO}_2^-$  production in activated microglia.

[0056] Figure 25 illustrates the anti-inflammatory effects of the KPV peptide, KPV dimer and prednisolone on edema induced in the hind paw of mice by the injection  $\alpha$ -carageenan as a function of time.

## DETAILED DESCRIPTION OF THE INVENTION

[0057] The references cited below are hereby incorporated by reference as if fully set forth herein.  $\alpha$ -MSH is a 13 amino acid, anti-inflammatory, anti-fungal peptide with the primary  
15 sequence SYSMEHFRWGKPV. In addition to its anti-fungal, anti-inflammatory properties, it also has anti-pyretic properties. The C-terminal trimer, KPV, appears responsible for these properties. Lipton, J.M., Antipyretic and Anti-inflammatory Lys-Pro-Val- Compositions and Methods of Use, U.S. Patent No. 5,028,592, issued July 2, 1991; Lipton, J.M., Antipyretic and Anti-inflammatory Lys-Pro-Val- Compositions and Methods of Use, U.S. Patent No. 5,157,023,  
20 issued October 20, 1992; Catania, A., Lipton J.M.,  $\alpha$ -Melanocyte Stimulating Hormone in the Modulation of Host Reactions, 14 *Endocr. Rev.*, 564-576 (1993); Lipton, J. M., Catania, A.,

Anti-inflammatory Influence of the Neuroimmunomodulator  $\alpha$ -MSH, 18 *Immunol. Today*, 140-145 (1997).

[0058] The core  $\alpha$ -MSH sequence (4-10) has learning, memory and behavioral effects but limited anti-pyretic and anti-inflammatory activity. Lipton, J.M., Catania, A., Anti-inflammatory Influence of the Neuroimmunomodulator  $\alpha$ -MSH, 18 *Immunol. Today*, 140-145 (1997).  $\alpha$ -MSH, the  $\alpha$ -MSH core and its tripeptide C-terminal have very low toxicity. Lipton, J.M., Catania, A., Anti-inflammatory Influence of the Neuroimmunomodulator  $\alpha$ -MSH, 18 *Immunol. Today*, 140-145 (1997).

[0059]  $\alpha$ -MSH is produced by the post translational processing of proopiomelanocortin and shares the 1-13 primary sequence with adrenocorticotrophic hormone (ACTH). Eberle, A. N., The Melanotropins, Karger, Basel, Switzerland (1988). It is secreted by a wide variety of cell types, including pituitary cells, monocytes, melanocytes, keratinocytes, epidermal cells and the epithelial cells of mucous membranes. Lipton, J.M., Catania, A., Anti-inflammatory Influence of the Neuroimmunomodulator  $\alpha$ -MSH, 18 *Immunol. Today*, 140-145 (1997); see also Catania et al., unpublished.

[0060]  $\alpha$ -MSH reduces inflammation and fever by modulating the inflammatory cascade locally and systemically. Rajora, N., Ceriani, G., Catania, A., Star, R.A., Murphy, M.T., Lipton, J.M.,  $\alpha$ -MSH Production, Receptors and Influence of Neopterin, in a Human Monocyte/macrophage Cell Line, 59 *H. Leukoc. Biol.*, 248-253 (1996); Star, R.A., Rajora, N. Huang, J., Stock, R.C., Catania, A., Lipton, J.M., Evidence of Autocrine Modulation of Macrophage Nitric Oxide Synthase by  $\alpha$ -MSH, 92 *Proc. Natl. Acad. Sci.*, 8016-8020 (1995);

Lipton, J.M., Ceriani, G., Macaluso, A., McCoy, D., Carnes, K., Biltz, J., Catania, A., Anti-inflammatory Effects of the Neuropeptide  $\alpha$ -MSH in Acute, Chronic and Systemic Inflammation, 741 *Ann. N.Y. Acad. Sci.*, 137-148 (1994); Rajora, N., Boccoli, G., Burns, D., Sharma, S., Catania, A., Lipton, J.M.,  $\alpha$ -MSH Modulates Local Circulating Tumor Necrosis Factor A in Experimental Brain Inflammation, 17 *J. Neurosci.*, 2181-2186 (1997); Richards, D.B., Lipton, J.M. Effect of  $\alpha$ -MSH (11-13)(Lys-Pro-Val) on Fever in Rabbits, 5 *Peptides*, 815-817 (1984); Hiltz, M.E., Lipton, J.M., Anti-inflammatory Activity of a COOH-terminal Fragment of the Neuropeptide  $\alpha$ -MSH, 3 *FASEB J.*, 2282-2284 (1989).

[0061] The broadest aspect of the invention is a composition and method of treatment of pathologies of the facial and maxillary sinuses having an inflammatory and/or fungal component. A preferred embodiment of the invention is composition for the treatment of sinusitis comprising a therapeutically effective amount of one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV in combination with a therapeutically effective amount of an antihistamine/decongestant selected from the group consisting of: pseudoephedrine, phenylephrine, phenylpropanolamine, chlorpheniramine, bromopheniramine, pheniramine and loratidine.

[0062] Another preferred embodiment of the invention is a composition for the treatment of sinusitis comprising a therapeutically effective amount of one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV in combination with a therapeutically effective amount of a corticosteroid selected from the group consisting of: betamethasone, cortisone, dexamethasone, hydrocortisone, methylprednisone, prednisone, and triamcinolone.

[0063] In another embodiment of the invention each of these compositions may comprise a therapeutically effective amount of an antibiotic selected from the group consisting of amoxicillin, ampicillin, azithromycin, erythromycin, nafcillin, penicillin, amoxicillin/clavulanate potassium, cefuroxime axetil, cefpodoxime proxetil, clarithromycin, and azithromycin.

5 [0064] In another embodiment of the invention each of these combination compositions may further comprise a therapeutically effective amount of a fungicide selected from the group consisting of itraconazole, econazole, ketoconazole, miconazole and fluconazole.

[0065] More preferably still, the peptides in each of these preferred combination compositions has the primary sequence of KPV or VPK-Ac-CC-Ac-KPV (Ac=Acetyl group). In all the preferred compositions, pharmacologically effective concentrations of the peptides may be as low as  $10^{-12}$ M but may be as high  $10^{-4}$  M.

10 [0066] In yet another embodiment of the invention, one or one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV, which may or may not be in combination with therapeutically effective amounts of antibiotics, corticosteroids, fungicides and/or antihistamine/decongestants is dissolved in a carrier. Formulations for solution or solids based drug delivery carriers are well known in the art. Such preferred carriers may be selected from the group consisting of saline, phosphate buffered saline, gelatin, maltodextrin, cellulose, microcrystalline cellulose, methyl cellulose and carboxymethyl cellulose.

15 20 [0067] The formulation of tablets are well known in the art. An exemplary formulation of a hard gelatinous tablet comprises:

Gelatine Bloom 30	70.0mg
Maltodextrin MD 05	108.0mg
di- $\alpha$ -tocopherol	2.0mg
Sodium ascorbate	10.0mg
Microcrystalline cellulose	48.0mg
Magnesium stearate	2.0 mg
Active Ingredient	$.2 \times 10^{-9}$ -. $2 \times 10^{-13}$ mg

An exemplary formulation of a hard tablet comprises:

Anhydrous lactose	130.5mg
Microcrystalline cellulose	80.0mg
di- $\alpha$ -tocopherol	2.0mg
Sodium ascorbate	10.0mg
Polyvinylpyrrolidone K30	5.0mg
Magnesium stearate	2.0mg
Active Ingredient	$.2 \times 10^{-9}$ -. $2 \times 10^{-13}$ mg

[0068] Another embodiment of the invention is a method for the treatment of sinusitis comprising topical or systemic administration of one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV. In another preferred method of the invention, one or more of these preferred peptides used in combination with therapeutically effective amounts of antihistamines/decongestants, corticosteroids, antibiotics, and/or fungicides, are topically or systemically applied to treat sinusitis.

[0069] In yet another embodiment of the invention, one or one or more peptides selected from the group of peptides with a C-terminal sequence consisting of KPV, HFRWGKPV, and SYSMEHFRWGKPV, which may or may not be in combination with therapeutically effective

amounts of antibiotics, corticosteroids, fungicides and/or antihistamine/decongestants, are topically or systemically applied before, during or after surgery to treat sinusitis.

[0070] The following examples teach the utility of  $\alpha$ -MSH as a fungicide and inflammatory. Methods in microbiology, molecular biology and biochemistry used but not explicitly described in this disclosure are amply described throughout the literature and well within the ability of one skilled in the art.

[0071] The peptides used in the following examples include:  $\alpha$ -MSH (1-13), (4-10), (6-13), and (11-13), all of which were N-acetylated and C-amidated, and ACTH (1-39) and (18-39) (CLIP). These peptides were prepared by solid-phase peptide synthesis and purified by reversed phased high performance liquid chromatography. Some examples also include a dimer of the amino acid sequence KPV, VPK-Ac-CC-Ac-KPV, which also was N-acetylated and C-amidated (KPV dimer). Dimers can be formed by adding cysteines at the N-termini of any of the above polypeptides and allowing the cysteines of two polypeptides to form a disulfide bond. Both homo-dimers and hetero-dimers can be formed using this method.

[0072] *C. albicans* (clinical isolate) was obtained from the collection of the Department of Microbiology, Ospedale Maggiore di Milano and maintained on Sabouraud's agar slants and periodically transferred to Sabouraud's agar plates and incubated for 48 hours at 28° C. To prepare a stationary growth phase yeast, a colony was taken from the agar plate and transferred into 30ml Sabouraud-dextrose broth and incubated for 72 hours at 32° C. Cells were centrifuged and suspended in Hank's balanced salt solution ("HBSS") to the desired concentration. Viability, determined by the exclusion of .01% methylene blue, remained >98%.



[0073] Statistical significance disclosed in the examples below was analyzed using one-way analysis of variance and the Student's t test. Probability values greater than 0.05 were considered significant.

#### EXAMPLE 1

5 [0074] Example 1 suggests that  $\alpha$ -MSH (11-13), (6-13) and (1-13) exhibit similar anti-fungal properties in general and similar anti-candidal properties specifically, as fluconazole over an exceedingly broad range of concentrations and therefore further suggests that  $\alpha$ -MSH (11-13), (6-13) and (1-13) may be therapeutic in treating fungal based sinusitis or sinusitis having a fungal component.

10 [0075] *C. albicans* ( $1 \times 10^6$ /ml in HBSS) was incubated in the presence or absence or  $\alpha$ -MSH (1-13) or (11-13) at concentrations in the range of  $10^{-15}$  M to  $10^{-6}$  M for 2 hours at 37° C. Cells were then washed in cold distilled water and diluted with HBSS to a concentration of 100 organisms/ml. One-ml aliquots were dispensed on blood agar plates and incubated for 48 hours at 37° C. Organism viability was estimated from the number of colonies formed.

15 [0076] In subsequent experiments using familiar procedures we compared activity of  $\alpha$ -MSH (4-10), (6-13), (11-13), ACTH (1-39), (18-39) and fluconazole, the latter an established antifungal agent. Melanocortin peptides and fluconazole were tested in concentrations of  $10^{-6}$  M to  $10^{-4}$  M. There were at least six replicates for each concentration of peptide.

20 [0077] Fig. 1 shows that *C. albicans* colony forming units (CFU) were greatly reduced by  $\alpha$ -MSH (1-13) and (11-13). Fig. 1 also shows that the VPK-Ac-CC-Ac-KPV peptide also

inhibited *C. albicans* colony formation. Concentrations of all three peptides from  $10^{-12}$ M to  $10^{-4}$ M had significant inhibitory effects on CFU ( $p < .01$  vs. control).

[0078] Fig. 2 demonstrates that in experiments comparing the relative potency of  $10^{-4}$ M melanocortin peptides in reducing *C. albicans* viability,  $\alpha$ -MSH (11-13), (6-13) and (1-13) were the most effective. Their inhibitory activity was similar to that of equimolar fluconazole. The core  $\alpha$ -MSH sequence (4-10), which has behavioral effects but little anti-inflammatory activity, caused approximately 50% inhibition of CFU. Fig. 2 also shows that although this inhibitory effect was substantial ( $p < .01$  vs. control), it was significantly less than that caused by  $\alpha$ -MSH fragments bearing the KPV signal sequence, i.e.,  $\alpha$ -MSH (6-13) and (11-13) ( $p < .01$ ), or the parent molecule  $\alpha$ -MSH (1-13) ( $p < .05$ ). ACTH (1-39) and the ACTH fragment (18-39) did not reduce *C. albicans* viability. Even higher concentrations of these ACTH peptides (up to  $10^{-4}$ M) were likewise ineffective in reducing *C. albicans* CFU (results not shown in the figures).

[0079] These results show that  $\alpha$ -MSH(1-13), its C-terminal tripeptide (11-13), and other  $\alpha$ -MSH fragments have significant fungicidal effects against *C. albicans*. The most effective of the  $\alpha$ -MSH peptides were those including the C-terminal amino acid sequence KPV of the  $\alpha$ -MHS sequence, i.e.,  $\alpha$ -MSH (1-13), (6-13) and (11-13). In addition, the sequence VPK-Ac-CC-Ac-KPV has also been shown to be at least as effective as  $\alpha$ -MSH (11-13) against microbes. The  $\alpha$ -MSH core sequence (4-10), which is known to influence learning and memory, but has little antipyretic and anti-inflammatory influence, was effective, but less so. The ACTH peptides (1-39) and (18-39) did not have significant candidacidal effects. These observations indicate that antifungal activity is not common to all melanocortin peptides, but rather is specific to  $\alpha$ -MSH amino acid sequences, and most particularly to the C-terminal

amino-acid sequences of  $\alpha$ -MSH. This strongly suggests that  $\alpha$ -MSH(1-13), its C-terminal tripeptide (11-13), and other  $\alpha$ -MSH fragments could server as a basis for a therapeutic treatment of sinusitis having a fungal component.

## EXAMPLE 2

5 [0080] Example 2 demonstrates that  $\alpha$ -MSH (1-13), (6-13) or (11-13) strongly inhibits Candidal germination. Accordingly, Example 2 also suggests that  $\alpha$ -MSH (1-13), (6-13) or (11-13) may be therapeutic in the treatment of sinusitis having in general a fungal component and specifically having a Candidal component. *C. albicans* from stationary phase cultures were washed twice with distilled water and suspended in HBSS to a final concentration of  $2 \times 10^6$ /ml. Hyphal growth was induced by addition of 10% inactivated horse serum (GIBCO/BRL, Great Britain) to yeast incubated for 45 minutes at 37° C with continuous shaking. Horse serum was removed by washing cells twice with HBSS and incubation was continued for 60 minutes at 37° C in the presence of  $\alpha$ -MSH (1-13), (6-13) or (11-13) at a concentration of  $10^{-6}$ M with continuous shaking. The percentage of filamentous cells was evaluated under a light microscope with the aid of hemocytometer. Experiments were run in triplicate and at least 200 cells were scored. Photomicrographs were taken with a MC100 camera attached to an Axioskop Zeiss microscope.

[0081] Figs. 3A-D show that coincubation of *C. albicans* with  $\alpha$ -MSH (1-13) or (11-13) inhibited germ tube formation induced by horse serum,  $\alpha$ -MSH (1-13) caused 28-32% reduction in the number of filamentous cells; the tripeptide inhibited germination by 54-58%. The octapeptide  $\alpha$ -MSH (6-13) had similar activity (approximately 50% inhibition)(not shown).

[0082] These results show that  $\alpha$ -MSH(1-13),  $\alpha$ -MSH(6-13), and  $\alpha$ -MSH(11-13) all significantly inhibit germination by *C. albicans*. The infection cycle of *C. albicans*, and candida in general, begins with adherence of fungal cells to epithelial cells. After adhesion, the fungal cells undergo a switch from ellipsoid blastophore form to various filamentous forms, including germ tubes, pseudohyphae, and hyphae. Gow, N.A., Germ Tube Growth of Candida Albicans, *Curr. Topics Med. Mycol.* **8**, 43 –45 (1997).  $\alpha$ -MSH peptides were added to *C. albicans* after promotion of hyphal growth.  $\alpha$ -MSH(1-13) inhibited subsequent germ tube formation by approximately 30%,  $\alpha$ -MSH(11-13) inhibited germ tube formation by approximately 56%, and  $\alpha$ -MSH(6-13) inhibited germ tube formation by approximately 50%. As with the results in example 1, the highest level of inhibition is observed for the tripeptide containing only the C-terminal KPV amino acid sequence. The results of example 2 suggest that  $\alpha$ -MSH(1-13), fragments of  $\alpha$ -MSH(1-13), or peptides containing the C-terminal KPV amino acid sequence of the  $\alpha$ -MSH peptides could be of therapeutic use not only in the prevention and treatment of early fungal infection as stated in example 1, but also in the treatment of later stage fungal infections. By preventing germination, the  $\alpha$ -MSH peptides could prevent the innervation of the epithelial cells that occurs during chronic candidiasis, thus providing a tool to combat long-term fungal infection, particularly fungal infection leading to sinusitis. This therapy would be especially beneficial to immunocompromised patients, who tend to exhibit a high rate of candidal infections.

### EXAMPLE 3

[0083] Example 3 illustrates that  $\alpha$ -MSH and its derivatives exhibit their anti-fungal properties in general and anti-candidal properties specifically, without compromising the ability of human neutrophils to independently combat Candida. Example 3 further suggests that systemic or topical administration of  $\alpha$ -MSH may used to treat fungal based sinusitis in general and candidal based sinusitis specifically. Venous blood (20ml) from health volunteers was anticoagulated with heparin. Neutrophils were isolated using dextran sedimentation and Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mi., USA) centrifugation. Erythrocytes were lysed via hypotonic shock. Neutrophils represented at least 97% of the cell suspension. Cell viability, estimated by trypan blue exclusion, was >98%. Neutrophils were suspended to a final concentration in HBSS.

[0084] *C. albicans* ( $1 \times 10^6$ ) were opsonized with human AB serum in a shaking water bath for 30 minutes at 37° C. Organisms were then incubated with neutrophils in medium or in medium with  $\alpha$ -MSH (1-13) or  $\alpha$ -MSH (11-13) in concentrations of  $10^{-15}$  M to  $10^{-4}$  M in a shaking water bath for 2 hours at 37° C. After incubation, the culture tubes were placed on ice to stop growth and extracellular organisms were washed twice with centrifugation at 1000 x g at 4° C. A 2.5% sodium desoxycholate solution was added to obtain a suspension of  $10^6$  cells/ml. Two 1/100 serial dilutions in HBSS were made to obtain a final suspension of 100 cells/ml. Aliquots of 1 ml were dispensed on blood agar plates and incubated for 48 hours at 37° C. Colony forming units (CFUs) were counted at the end of the incubation period. Experiments were run in triplicate and repeated using blood from 5 different donors.

[0085] Fig. 4 shows that  $\alpha$ -MSH(1-13) and (11-13) enhanced the killing of *C. albicans* by human neutrophils when administered in concentrations of  $10^{-12}$ M to  $10^{-4}$ M ( $p < .01$ ). Therefore, enhanced killing occurred over a very broad range of concentrations including picomolar concentrations, i.e. the quantity of  $\alpha$ -MSH found in human placenta. Catania, A., Airaghi, L., Garofalo, L., Cutuli, M., Lipton, J.M., The Neuropeptide  $\alpha$ -MSH in AIDS and Other Conditions in Humans, *Ann. N.Y. Acad. Sci.* **840**, 848-856 (1998).

[0086] Reduced killing of pathogens is a dire consequence of therapy with corticosteroids and nonsteroidal anti-inflammatory drugs during infection. Stevens, D.L., Could Nonsteroidal Anti-inflammatory Drugs (NSAIDS) Enhance Progression of Bacterial Infections to Toxic Shock Syndrome?, *Clin. Infect. Dis.*, **21**, 977-80 (1997); Capsoni, F., Meroni, P.L., Zocchi, M.R., Plebani, A.M., Vezio, M., Effect of Corticosteroids on Neutrophil Function: Inhibition of Antibody-dependent Cell-mediated Cytotoxicity (ADCC), *J. Immunopharmacol.* **5**, 217-230 (1983). This effect is particularly dangerous in immunocompromised patients.

[0087] These results show that  $\alpha$ -MSH(1-13) and  $\alpha$ -MSH(11-13) do not interfere with the ability of neutrophils to combat fungal infection. Presence of either peptide sequence, at concentrations ranging from  $10^{-12}$  M to  $10^{-4}$  M, enhanced killing of *C. albicans* by neutrophils. This is of therapeutic importance because many of the agents used to combat sinusitis, such as corticosteroids and nonsteroidal anti-inflammatories, have a detrimental effect on immune system function. This effect can be especially dangerous to immunocompromised patients. The results from example 3 suggest that the  $\alpha$ -MSH peptides can be utilized as antifungal agents (as discussed in example 1 and 2) without inhibiting the ability of neutrophils to combat bacteria and parasites.

#### EXAMPLE 4

[0088] Example 4 suggests a cellular mechanism to explain how  $\alpha$ -MSH exerts its anti-fungal properties and further suggests that systemic or topical administrators of  $\alpha$ -MSH may be used to treat fungal based sinusitis in general and candidal based sinusitis specifically. *C. albicans* ( $10^6$ /ml), permeabilized with toluene/ethanol, were incubated at 37° C with continuous shaking in the presence of  $10^{-6}$  M  $\alpha$ -MSH (1-13), (11-13), forskolin, an agent known to increase intracellular cAMP, or in medium alone. The reaction was stopped after 3 minutes by the addition of ice cold ethanol, cAMP was measured in duplicate using a commercial enzyme immunoassay (EIA) kit (Amersham, United Kingdom) after extraction via the liquid-phase method according to manufacturer's instructions. The effect of forskolin ( $10^{-6}$  M) on *C. albicans* colony formation was determined using the same procedure as for  $\alpha$ -MSH peptides.

[0089] Because many of the effects of  $\alpha$ -MSH are known to be mediated by induction of cAMP, we measured effects of  $\alpha$ -MSH peptides on cAMP accumulation in *C. albicans*. Fig. 5 shows that  $\alpha$ -MSH (1-13) and (11-13) enhanced cAMP content in the yeast. Fig. 6 shows the increase was of the same order of magnitude as that induced by equimolar forskolin, an adenylate cyclase activator. To determine whether increases in cAMP could be responsible for reduction in CFU, we tested the effects of forskolin on *C. albicans* viability. Results showed that  $10^{-6}$  M forskolin markedly inhibited *C. albicans* CFU relative to control ( $p < .01$ ). Fig. 6 demonstrates that the inhibitory effect was similar to that exerted by  $\alpha$ -MSH.

[0090] The mechanism of action of natural antimicrobial agents is only partly understood. Most of these peptides, including the defensins, alter membrane permeability and impair internal homeostasis of the organism. The first contact is made between the cationic

groups of the peptide and the negatively charged head of the target membrane. Then, the tertiary structure determines the mode of insertion of the peptide into membranes where they form ion channels or pores that disrupt cell integrity. It is known that cAMP-enhancing agents inhibit mRNA and protein synthesis in *C. albicans*. Bhattacharya, A., Datta, A., Effect of Cyclic AMP on RNA and Protein Synthesis in *C. albicans*, *Biochem. Biophys. Res. Commun.* **77**: 1483-44 (1977).

[0091] These results suggest that the  $\alpha$ -MSH peptides may exert their antifungal effect by increasing cAMP levels within the fungal cell.  $\alpha$ -MSH(1-13) and  $\alpha$ -MSH(11-13) both increased cAMP levels in *C. albicans*, with  $\alpha$ -MSH(11-13) having the larger effect of the two. The increase in cAMP caused by  $\alpha$ -MSH (11-13) was of the same order of magnitude as that caused by the adenylate cyclase activator forskolin. Forskolin was tested for its ability to inhibit *C. albicans* colony formation, and was shown to have an inhibitory effect between that of  $\alpha$ -MSH(1-13) and  $\alpha$ -MSH(11-13). The fact that forskolin inhibits fungal colony formation at a level similar to that of the  $\alpha$ -MSH peptides, coupled with the finding that the  $\alpha$ -MSH peptides increase cellular cAMP levels in a manner similar to forskolin, suggests that increased cAMP levels might be one mechanism by which  $\alpha$ -MSH exerts its antifungal effects. cAMP is formed via a G protein mediated pathway. G proteins are activated when a signaling molecule binds to a cell surface receptor, triggering a conformational change in the receptor. This conformational change causes the  $\alpha$  subunit of the G protein to release GDP and bind GTP, forming the  $\alpha$ -GTP subunit that activates adenylate cyclase. Adenylate cyclase then converts ATP to cAMP, which activates enzymes such as protein kinase A and causes the phosphorylation of a variety of downstream targets. It is believed that  $\alpha$ -MSH exerts its effects by binding to cell surface



receptors that activate G proteins, or at any other step along the G protein pathway. Agents that increase adenylate cyclase activity and boost cellular cAMP levels have been shown to inhibit both mRNA synthesis and protein translation in *C. albicans* by phosphorylating downstream targets.

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#### EXAMPLE 5

[0092] Example 5 suggests that systemic or topically administered  $\alpha$ -MSH may be useful in reducing the inflammation associated with sinusitis. Example 5 further suggests that such applications of  $\alpha$ -MSH would be clinically therapeutic for the treatment of sinusitis. The anti-inflammatory activity of the tripeptide was demonstrated through the use of an animal model developed by Sparrow and Wilhelm (1957), J. Physiol., 137:51-65. This model relies on the principle that localized, subcutaneous injections of histamine will result in a localized increase in capillary permeability. When the test animal has been pretreated with blue dye intravenously, the localized histamine injections will elicit blue-colored "weals" around the injection site. Thus, by preadministration of an effective anti-inflammatory agent the blue color of the histamine-induced weals will be much less pronounced, with the amount of color reduction being dependent on the relative amount and/or potency of the anti-inflammatory agent used.

[0093] Non-moulting New Zealand white rabbits were used for the Sparrow/Wilhelm assay. The skin of the rabbits back was closely clipped 1-2 days previous to the experiment, but not depilated, and the rabbits were kept warm until tested. Various amounts of the protected tripeptide Ac-Lys-Pro-Val-NH<sub>2</sub> were injected intravenously into an ear vein approximately 15 minutes prior to intravenous injection of blue dye. Control rabbits received sham injections.

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Fifteen minutes following injection of the agent or sham, the rabbits received approximately 30 mg/kg of Pontamine blue dye as a 2.5% solution in 0.45% saline, into an exposed vein. Immediately following dye injections, histamine was injected intradermally in a 0.10 ml volume (1.25 mg histamine 0.1 ml volume) at several sites on each side of the spine. In all, one vertical row of six injections were made on each side of the spine. The relative intensity of the resultant blue weals were scored by an independent observer 30 minutes after histamine injection. The results are displayed in Table I below.

TABLE I

## Anti-inflammatory Activity of the Tripeptide

No Animals Tested	Tripeptide Dose +	Result
3 (2E, 1C) *	5	E lighter than C
2 (1E, 1C)	10	E lighter than C
2 (1E, 1C)	5	E lighter than C
2 (1E, 1C)	1.25	E lighter than C
2 (1E, 1C)	0.625	No difference observed

\* E = experimental; C = control

+ Dosages in ug of protected tripeptide per kg body weight, administered intravenously.

[0094] As will be appreciated from the results displayed in Table I, intravenous doses down to 1.25 ug tripeptide per kg body weight resulted in an appreciable reduction in histamine-induced blue weal formation and is thus indicative of an effective anti-inflammatory action. At doses of 5 and 10 ug/kg, the observed response was even more pronounced. Also as will be appreciated, the anti-inflammatory effect of the tripeptide is observed at relatively lower doses as compared to its anti-pyretic effect.

[0095] These results show that a peptide containing the KPV amino acid sequence of  $\alpha$ -MSH has anti-inflammatory properties. Rabbits injected with histamine after injection of a blue marking dye will exhibit blue weal formation around the injection site. This occurs because histamine increases blood vessel permeability, allowing the dye to seep into the injection area.

5 When the rabbits were pre-injected with as little as 1.25  $\mu$ g/kg body weight of a protected KPV tripeptide, the histamine-induced weal exhibited a substantially less intense blue color. This suggests that the KPV tripeptide is interfering with the ability of histamine to increase blood vessel permeability. Histamine plays a central role in the inflammation reaction by dilating blood vessels, increasing vessel permeability, and increasing blood flow. Histamine also triggers the release of cytokines by leukocytes, which further increases the inflammation reaction. The ability of the KPV tripeptide to block histamine function suggests that peptides containing the  $\alpha$ -MSH KPV amino acid sequence could serve as potent anti-inflammatory agents.

#### EXAMPLE 6

[0096] Example 6 still further suggests that the anti-inflammatory properties of  $\alpha$ -MSH may be clinically therapeutic for the treatment of sinusitis and may serve as substitute for corticosteriod based treatment regimens. A second in vivo bioassay for anti-inflammatory activity was conducted in which the action of the tripeptide was compared to that of hydrocortisone. In this assay, the two agents were given at similar doses and tested for their

20 independent ability to inhibit carrageenan-induced swelling in rat paws. This assay, the rat paw edema test, was conducted generally as it is typically performed in the art, for example, as described by Winter et al. (1962), Proc. Soc. Exp. Biol. Med., 111:544 or in U.S. Pat. No. 4,150,137.

[0097] Briefly, the assay was performed as follows. Each of twenty-four male Sprague-Dawley rats was assigned to one of four groups: Tripeptide treatment and controls (matched according to body weight and initial paw volume), hydrocortisone treatment and matched controls. The volume of the right rear paw of the test and control animals was determined using standard procedures and a mercury displacement volumetric technique. An intraperitoneal injection of the tripeptide (Ac-Lys-Pro-Val-NH<sub>2</sub>, 100 mg/kg, N=6), of hydrocortisone (100mg/kg, N=6), or saline (matched volume, N=12) was given to each rat. One hour later 0.5 ml of 1% lambda carrageenan in saline solution was injected into the right rear paw of the animals and the paw volume was again recorded (baseline measure). For comparison of the effects of the two treatments, paw volume of experimental animals measured at hourly intervals was expressed as a percentage of the volume change of their respective matched controls.

[0098] The results of this experiment are shown in Fig. 7. As will be appreciated from this data, except for the first hour when hydrocortisone markedly inhibited swelling ( $p < 0.05$ , Mann-Whitney test), there was no significant difference in the inhibition of paw edema caused by the tripeptide and hydrocortisone ( $p < 0.20$ ).

[0099] These results show that a peptide containing the KPV amino acid sequence of  $\alpha$ -MSH could serve as a potential replacement for corticosteroids in the treatment of inflammation.  $\alpha$ -MSH(11-13) was compared to hydrocortisone for its ability to prevent carrageenan-induced rat paw inflammation. The development of swelling after carrageenan injection is a biphasic event. The first phase, occurring approximately 1 hour after injection, is attributed to the release of histamine and serotonin. The second phase is attributed to the release of prostaglandin-like substances, and is sensitive to both steroidal and non-steroidal anti-inflammatory agents.

Prostaglandins are produced from arachidonic acid by cyclooxygenase, and like histamine they serve to increase blood vessel permeability. During the first hour after carrageenan injections, hydrocortisone caused approximately a 30% decrease in paw inflammation, while no such reduction was observed in the presence of  $\alpha$ -MSH(11-13). Between 1 and 4 hours, however,  $\alpha$ -MSH(11-13) and hydrocortisone exhibited nearly identical anti-inflammatory activity. Corticosteroids such as hydrocortisone inhibit inflammation by preventing the release of arachidonic acid from phospholipids, which in turn inhibits the formation of prostaglandins. While example 5 suggested that the  $\alpha$ -MSH tripeptide inhibits inflammation by blocking histamine function, example 6 suggests that  $\alpha$ -MSH may also inhibit inflammation via a mechanism similar to that of the corticosteroids. It is also believed that  $\alpha$ -MSH serves to inhibit inflammation by increasing the formation of endogenous corticosteroids. These substances are up-regulated by ACTH, which like  $\alpha$ -MSH is a melanocortin peptide. Regardless of the exact mechanism of  $\alpha$ -MSH anti-inflammatory activity, these results suggest that peptides containing the C-terminal  $\alpha$ -MSH amino acid sequence KPV have potential therapeutic utility in the treatment of inflammation, particularly inflammation tied to sinusitis.

#### EXAMPLE 7

[0100] Example 7 suggests that  $\alpha$ -MSH and its derivatives may be employed in general to reduce inflammation and in particular may be employed to reduce inflammation associated with sinusitis. Hiltz, M.E., Catania, A., Lipton, J.M., Anti-Inflammatory Activity of  $\alpha$ -MSH(11-13) Analogs: Influence of Alteration in Stereochemistry, 12 Peptides 767 (1981). More particularly Example 7 suggests that the COOH-terminal tripeptide of  $\alpha$ -MSH has potent anti-

inflammatory properties and further suggest that alterations in amino acid chirality tripeptide can markedly affect the peptides  $\alpha$ -MSH anti-inflammatory properties. Female BALB/C mice (Simonsen Laboratories), 7 weeks old, were housed at 23-25°C in groups of no more than 5 per cage [28 cm (L) x 18 cm (W) x 13 cm (H)]. They were allowed to acclimatize to standard lighting and temperature conditions for at least 1 week, with food and water available ad lib, before the experiment began. Five to ten animals were randomly assigned to each treatment group each test day, and all experiments were repeated at least twice to confirm reproducibility. To reduce error due to slight differences in responses to the irritant of animals from different shipments, tests of each agent were performed as separate experiments in which experimental and control animals were drawn from the same shipment.

**[0101]** In the experiments each animal was anesthetized with 1 mg of pentobarbital sodium solution (50 mg/kg, Nembutal, Abbott Laboratories, Abbott park, IL). Baseline ear thickness was measured for both ears with a spring-loaded micrometer (Swiss Precision Instruments, Los Angeles, CA). Ear thickness was expressed in units of  $10^{-3}$  cm; the average thickness of unstimulated ears was approximately  $25-30 \times 10^{-3}$  cm. After baseline measurements were taken, one of six solutions was injected IP. Control animals received 0.2 ml of sterile saline, and experimental animals received Ac- $\alpha$ -MSH(11-13)-NH<sub>2</sub>, i.e., Ac-Lys-Pro-Val-NH<sub>2</sub>), Ac[D-Lys<sup>11</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub>, Ac[D-Pro<sup>12</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub>, Ac[D-Val<sup>13</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> or Ac-[D-Lys<sup>11</sup>, D-Val<sup>13</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> in the same volume of saline. All of the peptides were custom synthesized by Peninsula Laboratories (Belmont, CA) and were determined to be both pure by HPLC and to possess the expected amino acid analytical composition. The doses of the tripeptides were 10  $\mu$ g ( $2.6 \times 10^{-8}$  M), 20  $\mu$ g ( $5.2 \times 10^{-8}$  M), 40

$\mu\text{g}$  ( $1.04 \times 10^{-7}$  M), and  $80 \mu\text{g}$  ( $2.08 \times 10^{-7}$  M). Immediately after saline or  $\alpha$ -MSH injection, a micropipette was used to coat both sides of each ear with  $10 \mu\text{l}$  ( $40 \mu\text{l}$  per mouse) of a 0.5% picryl chloride (Polysciences, Warrington, PA) solution in acetone.

[0102] Ear thickness was remeasured 3 and 6 h after picryl chloride application.

5 Swelling was determined by subtracting baseline thickness from the measurements for each ear at 3 and 6 h. The differences for both ears were then averaged for the final analysis. On rare occasions when swelling did not occur in the control animals, the data for that day were considered invalid and were not used in the final analysis.

[0103] Repeated-measure ANOVA techniques were used to determine if there was an overall difference among group data for each peptide tested in separate experiments. In those cases in which the ANOVA yielded significant results, Dunnett's test was then used to compare the effects of peptide doses with control (saline) data for each of the two time periods, 3 and 6 h.

[0104] Fig. 8 illustrates inhibition by  $\alpha$ -MSH (11-13) of inflammation induced in the ear of a mouse induced by picryl chloride application. Ac- $\alpha$ -MSH(11-13)-NH<sub>2</sub> inhibited acute inflammation. The ANOVA showed a highly significant dose effect,  $F(4,92) = 9.35$ ,  $p < 0.0001$ . There was also a significant time effect,  $F(1,92) = 40.8$ ,  $p < 0.001$ , but no significant interaction. The average swelling in the controls was significantly greater than in any of the peptide treatment groups at both 3 and 6 h ( $p < 0.05$ , Dunnett's test, two-tail).

[0105] Fig. 9 illustrates that Ac[D-Lys<sup>11</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> likewise significantly reduced inflammation [ $F(4,135) = 7.83$   $p < 0.0001$ ] in the ear of a mouse induced by picryl chloride application. Ear swelling after all doses of peptide was less than control ( $p < 0.05$ ).

There was a significant time effect,  $F(1,135) = 43.8$ ,  $p < 0.001$ , but no significant dose-time interaction. Fig. 10 illustrates that Ac[D-Pro<sup>12</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> had no significant effect on inflammation [ $F(4,139) = 0.62$ ,  $p > 0.65$ ]. By contrast, Fig. 11 illustrates that Ac[D-Val<sup>13</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> had a marked inhibitory influence [ $F(4,136) = 19.6$ ,  $p < 0.001$ ]. There was a significant time effect in the Ac[D-Val<sup>13</sup>] experiment,  $F(4,138) = 3.96$ ,  $p < 0.005$ , likely due to increases over time for the control, 10 and 80  $\mu$ g groups and little change for the 20 and 40  $\mu$ g groups. Dunnett's test applied to control and treatment data for the 3- and 6-h time periods indicated that the control swelling was significantly greater than in the peptide treatment groups ( $p < 0.01$ ).

[0106] As Fig. 12 illustrates, Ac-[D- Lys<sup>11</sup>, D-Val<sup>13</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> showed a significant overall reduction in inflammation [ $F(4,102) = 19.8$ ,  $p < 0.0001$ ], and a significant time effect,  $F(1,102) = 73.2$ ,  $p < 0.0001$ , but no significant interaction. Treatment means were all significantly ( $p < 0.01$ ) less than control values for both the 3- and 6-h measurements.

[0107] As is shown in Table 2, the results of the experiments were converted to percent inhibition of swelling for comparisons of the effectiveness of the peptide conformations. The two peptides with D-Val<sup>13</sup> conformation induced more consistent, large anti-inflammatory effects at 3 and 6 h than did the other tripeptides. Although in these experiments, the well-known bell-shaped influence of peptides complicated the comparisons, the D-Val<sup>13</sup> tripeptides were generally more potent and their effects were sustained in the 6-h measurement. D-Lys<sup>11</sup> substitution did not increase the potency or duration of anti-inflammatory activity and the strong action of the largest does of Ac- $\alpha$ -MSH(11-13)-NH<sub>2</sub> suggests that its actions rank roughly third among those recorded. The lack of significant effect on inflammation of the Ac [D-Pro<sup>12</sup>]  $\alpha$ -



MSH(11-13)-NH<sub>2</sub> indicates that the L-Pro configuration is likely central to the anti-inflammatory activities of the molecule.

**Table 2**

	% Inhibition		Amino Acid Analysis	% Purity
	3 h	6 h		
Ac- $\alpha$ -MSH(11-13)-NH <sub>2</sub>			K(0.99), P(1.01), V(0.94)	>99
10 $\mu$ g	26	25		
20 $\mu$ g	21	19		
40 $\mu$ g	33	26		
80 $\mu$ g	50	42		
Ac-[D-Lys <sup>11</sup> ] $\alpha$ -MSH(11-13)-NH <sub>2</sub>			K(0.97), P(1.03), V(0.94)	>93
10 $\mu$ g	23	17		
20 $\mu$ g	25	16		
40 $\mu$ g	35	37		
80 $\mu$ g	34	29		
Ac-[D-Pro <sup>12</sup> ] $\alpha$ -MSH(11-13)-NH <sub>2</sub>			K(0.99), P(1.01), V(0.97)	>93
10 $\mu$ g	4	8		
20 $\mu$ g	11	5		
40 $\mu$ g	10	8		
80 $\mu$ g	8	6		
Ac-[D-Pro <sup>13</sup> ] $\alpha$ -MSH(11-13)-NH <sub>2</sub>			K(0.99), P(1.01), V(0.97)	>96
10 $\mu$ g	24	23		
20 $\mu$ g	48	54		
40 $\mu$ g	39	45		
80 $\mu$ g	41	29		
Ac-[D-Lys <sup>11</sup> , D-Val <sup>13</sup> ] $\alpha$ -MSH(11-13)-NH <sub>2</sub>			K(1.04), P(0.91), V(1.05)	>99
10 $\mu$ g	22	22		
20 $\mu$ g	55	46		
40 $\mu$ g	42	41		
80 $\mu$ g	40	44		

[0108] Altering the stereochemical make-up of the tripeptide via D-substitution had two major effects: increased potency in the case of Ac[D-Val<sup>13</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> and Ac-[D-Lys<sup>11</sup>, D-Val<sup>13</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub> and reduced the anti-inflammatory activity in Ac[D-Pro<sup>12</sup>]  $\alpha$ -MSH(11-13)-NH<sub>2</sub>.

5 [0109] These results again show the ability of the  $\alpha$ -MSH(11-13) tripeptide to inhibit inflammation, as well as illustrating the importance of chirality to the effectiveness of the peptide.  $\alpha$ -MSH(11-13) reduced mouse ear swelling in the presence of the skin irritant picryl chloride at doses as low as  $2.6 \times 10^{-8}$  M. At the highest  $\alpha$ -MSH(11-13) dosage,  $2.08 \times 10^{-7}$  M, inflammation was reduced by 50% 3 hours after picryl chloride treatment. The anti-inflammatory effect of  $\alpha$ -MSH(11-13) was reduced after 6 hours, but there was still significantly less ear swelling than in the control mice. When the chirality of the amino acids making up the  $\alpha$ -MSH tripeptide was switched from the L configuration to the D configuration, there was a direct effect on peptide activity. Switching the proline residue to the D-form caused the most marked change, completely destroying the ability of the tripeptide to inhibit inflammation. Switching the lysine residue to the D-form created a tripeptide that was similar to the L-form version in its ability to inhibit inflammation at low concentrations, but not as effective at higher concentrations or over longer time periods. Switching the valine residue to the D-form, either alone or in conjunction with the lysine residue, created a tripeptide that was more effective at inhibiting inflammation than the standard L-form version. This peptide inhibited inflammation  
20 at lower concentrations than the D-form peptide, and was effective over a longer time period and suggests that the use of such peptides might serve to prevent unwanted side effects in a therapeutic setting by making it less likely that the peptide will interact with other compounds.

EXAMPLE 8

[0110] Example 8 suggests that  $\alpha$ -MSH reduces inflammation through both centrally and peripherally mediated mechanisms and accordingly, systemic or topical administration of  $\alpha$ -MSH may be therapeutically effective in the treatment of sinusitis. See Macaluso, A., McCoy, D., Ceriani, G., Watanabe, T., Biltz, J., Catania, A., and Lipton, J.M., Anti-inflammatory Influences of  $\alpha$ -MSH Molecules: Central Neurogenic and Peripheral Actions, 14(4), J. of Neuroscience 2377-2382 (1994). Female BALB/c mice (Simonsen Laboratories, Gilroy, CA), 7-8 weeks old, were housed at 23-25°C in groups not exceeding five animals per cage [28 cm (L) x 18 cm (W) x 13 cm (H)]. Before the experiments they were acclimatized for not less than 1 week before experimentation to standard lighting and temperature conditions with food and water freely available.

[0111] Mice were anesthetized with 10% pentobarbital sodium solution (1 mg/mouse, 50 mg/ml, Nembutal sodium solution; Abbott Laboratories, North Chicago, IL). Baseline ear thickness measurements were taken with a spring-loaded micrometer (Swiss Precision Instruments, Los Angeles, CA). Ear thickness was expressed in centimeters and was measured at least twice at each time point. The average thickness of unstimulated ears was 26.82 cm<sup>3</sup>. Inflammation was induced by injecting 20  $\mu$ l of recombinant human IL-1 $\beta$  (1500 U; Genzyme, Cambridge, MA) directly into the skin (intradermally) of one ear of each mouse using a 28 gauge needle (Hiltz et al., 1992). Measures of ear thickness were repeated 4 and 6 hr later, while the mice were under sodium pentobarbital anesthesia. Edema was assessed by subtracting baseline measures from 4 and 6 hr readings for each animal.  $\alpha$ -MSH<sub>1-13</sub> (1663.9 gm/mol; Peninsula Laboratories, Belmont, CA) or  $\alpha$ -MSH<sup>11-13</sup> (383.48 gm/mol; Peninsula Laboratories) dissolved

in saline was injected intracerebroventricularly (20  $\mu$ l) using procedures described previously (Lipton et al., 1991; Lipton and Catania, 1993).

[0112] Several agents were administered at the time of ear challenge to pharmacologically block certain receptors: atropine (Sigma Chemical Co.; 150  $\mu$ g i.p.), a muscarinic receptor blocker that acts on autonomic end-organ receptors, was chosen because of links between inflammation and modulation of pain and the finding that muscarinic antagonists increase pain threshold (Hartvig et al., 1989); phentolamine (Sigma; 150  $\mu$ g i.p.) an agent that competes for occupancy of  $\alpha$ -adrenergic receptors; propranolol (Sigma; 150  $\mu$ g, i.p.; 30  $\mu$ g, i.c.v.), a nonspecific competitive antagonist for  $\beta$ -adrenergic receptors. After positive effects were observed with propranolol, selective antagonists of  $\beta$ -adrenergic receptors were tested; atenolol (Sigma; 150  $\mu$ g, i.p.), a  $\beta_1$ -adrenergic receptor antagonist; butoxamine (Sigma; 150  $\mu$ g, i.p.), a  $\beta_2$ -adrenergic receptor blocker.

[0113] Because surgical dissection of trigeminal structures that innervate the ear of the mouse is very difficult, tests to learn whether descending neural pathways are essential to the anti-inflammatory activity of centrally administered  $\alpha$ -MSH peptides were performed in mice with inflammation induced in a hind paw. In these experiments, each animal was anesthetized with pentobarbital sodium solution as above. Baseline foot pad thickness of both hind paws was measured with a spring-loaded micrometer (Swiss Precision Instruments, Los Angeles, CA). Paw thickness was expressed in units of  $10^{-3}$  cm; average thickness of unstimulated paws was  $171.5 \times 10^{-3}$  cm across shipments of animals. Kappa carrageenan (Sigma) dissolved in saline (0.05%, 20  $\mu$ l) was injected (28 gauge allergy test syringe) into one footpad, and saline (20  $\mu$ l) was injected into the other. For the analyses, the increase in thickness of the control saline-

injected paw of each animal was subtracted from that of the carrageenan-injected paw, to eliminate the influence of mechanical injury and volume of the injected fluid. To be certain that mice with severed spinal cords can react to anti-inflammatory agents, prednisolone (2.5 mg/mouse i.p.) was administered to 10 mice with spinal transection and inflammation induced by carrageenan. Treatment with the locally acting steroid reduced swelling up to 29% (average, relative to saline controls) at 4.5 hr. after carrageenan. This finding indicates that spinal transection in these mice does not result in stasis of inflammation that is unalterable as a result of vasodilation, hypotension, or other hemodynamic changes.

[0114] Pilot studies indicated that interruption of the spinal cord of the mouse by surgical exposure, visualization, and severing with a scalpel blade resulted in marked bleeding, morbidity, and death in a substantial number of animals. However, a standardized crushing of lumbar vertebrae with a hemostat (Kelly) was effective in severing the cord; no deaths were caused by this treatment. In the experiments proper, all animals were tested after cord section when the anesthesia had worn off: none showed behavioral or motor responses to pinching of the hind feet with a hemostat; all had complete paralysis of the hind limbs. Data of animals that did not meet these criteria were excluded from the analyses. The experiments were limited to 4.5 hr to reduce problems of bowel and bladder dysfunction. Immediately after the 4.5 hr measurements all mice were killed with an overdose of sodium pentobarbital.

[0115] Because of the size of the experiments and the requirement for several shipments of animals over time, several experiments were performed, each with complete treatment and control groups drawn from the same shipment of mice. Omnibus analysis of variance techniques (Dynastat, Washington, DC) were used to test overall differences among group means. Tukey's

protected *t* test was used to compare individual means. Probability values of  $<0.05$  were considered significant.

[0116] In order to determine whether  $\alpha$ -MSH anti-inflammatory properties are centrally or peripherally mediated, propranolol, a  $\beta$ -receptor blocker, was administered centrally and peripherally. As is shown in Fig.13, when propranolol was injected centrally there was little effect on the anti-inflammatory influence of  $\alpha$ -MSH (1-13) (inhibition by  $\alpha$ -MSH was 36% and 32% at 4 hr and 6 hr respectively). By contrast when 300  $\mu$ g of propranolol was administered intraperitoneally, the anti-inflammatory response of  $\alpha$ -MSH was inhibited. This indicates that competitive binding of peripheral  $\beta$ -receptors inhibits the anti-inflammatory influence of centrally administered  $\alpha$ -MSH. Subsequent tests determined the role of which  $\beta$ -adrenergic receptors were connected with the anti-inflammatory effect of centrally administered  $\alpha$ -MSH. As is shown in Fig. 14, when atenolol, a  $\beta_1$  adrenergic antagonist was injected IP, there was little inhibition of  $\alpha$ -MSH (1-13)'s anti-inflammatory activity. By contrast, when the  $\beta_2$ -receptor antagonist, butoxamine, was similarly administered, the  $\alpha$ -MSH(1-13)'s anti-inflammatory effects were significantly reduced. These results indicate that  $\beta_2$ -receptor activity in the periphery is essential to the anti-inflammatory action of  $\alpha$ -MSH. As is shown in Fig. 15, blockade of  $\alpha$ -adrenergic receptors with phentolamine, and cholinergic (muscarinic) receptors with atropine, did not alter the anti-inflammatory effect of  $\alpha$ -MSH (1-13).

[0117] As shown in Fig. 16, central administration of  $\alpha$ -MSH (1-13) in spinal cord intact mice markedly inhibited inflammation induced in the hind paw. The greatest effect was at 3 hr (57% inhibition); inhibition was less at 4.5 hr (29%). By contrast, spinal cord transection

significantly reduced the anti-inflammatory effect of centrally administered  $\alpha$ -MSH (1-13). As is shown in Fig. 17, although transection of the spinal cord significantly reduced the early anti-inflammatory effect of  $\alpha$ -MSH (1-13) given intraperitoneally, there was a significant, although smaller, inhibitory effect later in the period.

5 [0118] To determine if the influence of systemically administered  $\alpha$ -MSH requires it to act on the brain to induce descending inhibitory signals,  $\alpha$ -MSH (1-13) was administered IP to mice with spinal cord transection. Fig. 18 suggests that the tripeptide had a marked inhibitory effect on inflammation.

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10 [0119] These results show that the  $\alpha$ -MSH peptides reduce inflammation through both centrally and peripherally mediated mechanisms. The effect of intracerebroventricularly-injected  $\alpha$ -MSH(1-13) on IL-1 $\beta$ -induced mouse ear inflammation was tested in the presence of several receptor-blocking agents. When mice were centrally injected with propranolol, a  $\beta$ -receptor blocker, there was little change in the anti-inflammatory effects of  $\alpha$ -MSH(1-13). However, when propranolol was injected peripherally, there was a substantial decrease in the anti-inflammatory effectiveness of  $\alpha$ -MSH(1-13). Injection of the mice with an  $\alpha$ -adrenergic receptor antagonist (phentolamine) or a cholinergic receptor antagonist (atropine) had no effect on  $\alpha$ -MSH(1-13) activity, regardless of whether these were injected centrally or peripherally. This suggests that centrally injected  $\alpha$ -MSH(1-13) exerts its anti-inflammatory effects through a peripheral  $\beta$ -receptor pathway. To determine which  $\beta$ -receptors were involved,  $\alpha$ -MSH(1-13) activity was measured in the presence of either a  $\beta_1$ -adrenergic receptor antagonist (atenolol) or a  $\beta_2$ -receptor antagonist (butoxamine). Atenolol had little effect on the anti-inflammatory activity

of  $\alpha$ -MSH(1-13), while butoxamine reduced this activity substantially. This suggests that it is the  $\beta_2$  receptors of the peripheral nervous system that mediate the anti-inflammatory effect of  $\alpha$ -MSH(1-13).

[0120] The anti-inflammatory effect of  $\alpha$ -MSH was next measured in mice with transected spinal cords. Centrally injected  $\alpha$ -MSH(1-13) greatly reduced carrageenan-induced paw swelling in mice with intact spinal cords, but had no effect on paw swelling in transected mice. Peripherally injected  $\alpha$ -MSH(1-13), on the other hand, reduced swelling in both intact and transected mice, although the effect was not as marked in the transected mice and took longer to occur. Peripherally injected  $\alpha$ -MSH(11-13) was even more effective at reducing swelling in transected mice, displaying a level of inhibition similar to that seen in intact mice treated with  $\alpha$ -MSH(1-13). These results suggest that the  $\alpha$ -MSH peptides do not have to act on the brain to produce descending inhibitory signals, meaning that either topical or systemic administration of  $\alpha$ -MSH peptides should be effective in treating inflammation, especially inflammation related to sinusitis.

#### EXAMPLE 9

[0121] Example 9 suggests that the anti-inflammatory peptides of  $\alpha$ -MSH may be associated with reducing the cytokines connected to the inflammatory response including IL-6 and TNF- $\alpha$  and NO. See Delgado, R., Carlin, A., Alraghi, L., Demitri, M., Meda, L., Galimberti, D., Pierluigi, B., Lipton, J.M., Catania, A., Melanocortin Peptides Inhibit Production of Proinflammatory Cytokines and Nitric Oxide by Activated Microglia, 63 J. of Leukocyte Biol. 740 (1998). This further suggests the  $\alpha$ -MSH may be the therapeutically effective, when administered topically or systemically for the treatment of sinusitis. The N9 clone of murine



microglial cells was obtained by immortalization of embryonic brain cultures with the 3RV retrovirus carrying an activated v-myconcogene. N9 cells were cultured in T-75-cm<sup>2</sup> culture flasks (Corning, Cambridge, MA) and maintained at 37°C in a humidified incubator under 5% CO<sub>2</sub> atmosphere in RPMI 1640 supplemented with 2mM L-glutamine, 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate (GIBCO-BBL, Paisley, UK), and 10% heat-inactivated fetal bovine serum (FBS, Hyclone Lab, Inc., Logan, UT) until experiments were performed. Cells were used between the first and the tenth passage.

[0122] Sub-confluent microglial cells were washed twice with phosphate-buffered saline (PBS) and incubated with trypsin 0.025% and ethylenediaminetetraacetate (EDTA) 0.02% without calcium and magnesium for 3 min. at 37°C to detach the cells from the culture flask. Cells were then resuspended in medium and incubated in 24-well tissue-culture plates at a concentration of 2 x 10<sup>5</sup> cells/mL for 16 h in a humidified incubator (37°C, 5% CO<sub>2</sub>). Growth medium was removed and cell monolayers were stimulated with 10 ng/mL lipopolysaccharide (LPS, from *Escherichia coli* 055:B5, Sigma Chemical Co., St. Louis, MO) plus 1 U/ml, murine IFN-γ (Sigma). To test effects of melanocortin peptides, concentrations (1, 10, 25, 50 and 100 μM) of α-MSH (1-13), α-MSH (11-13), and ACTH (1-24) (Sigma) were dissolved in medium and added to wells 10 min before treatment with LPS + IFN-γ. Although production of TNF-α and NO was reduced to even much lower concentrations of the peptides, concentrations in the micromolar range had the most profound and consistent inhibitory effects. Although lower concentrations were more effective in previous research, we elected to use micromolar concentrations in the present studies because they were more effective for the experimental conditions (cell type, incubation period, concentration of the stimuli). Cell-free supernatants were harvested after 24 h incubation and assayed for TNF-α, IL-6 and NO<sub>2</sub>. Viability of cells

was assessed by trypan blue exclusion for each experimental condition: it was consistently >98%. Dexamethasone (Sigma) and N-monomethyl-L-arginine (L-NMMA: 100  $\mu$ M, Cayman Chemical, Ann Arbor, MI) were used as positive controls for TNF- $\alpha$  and NO inhibition, respectively. Tests were repeated in at least three independent experiments and assays were performed in triplicate.

[0123] TNF- $\alpha$  bioactivity was measured in supernatants of cell cultures by standard cytotoxicity assay using L929 cells and recombinant human TNF- $\alpha$  (Sigma) as standard. The detection limit of the bioassay was 20 pg/mL. IL-6 was measured using a commercial murine enzyme-linked immunosorbent assay (ELISA: RPN 2714, Amersham, Little Chalfont, UK).

[0124] NO is rapidly oxidized to nitrite in culture medium, and nitrite ( $\text{NO}_2^-$ ) concentration is an indicator of NO production. Cell-free culture supernatants were mixed with equal amounts of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamide in 2.5% phosphoric acid) in wells of 96-well ELISA plates. Samples were incubated at room temperature for 10 min and absorbance was measured at 540 nm with the use of a microplate reader. Nitrite concentrations were calculated using a sodium nitrite standard curve.

[0125] cAMP accumulation in N9 cells was measured as previously described. Briefly, cells in six-well plates were co-incubated at 37°C with (1) medium; (2) forskolin (100  $\mu$ M); (3) LPS (10 ng/mL) + IFN- $\gamma$  (1 U/mL); (4)  $\alpha$ -MSH (1-13) or  $\alpha$ -MSH (11-13) (1, 10, 50  $\mu$ M); (5) LPS + IFN- $\gamma$  and either  $\alpha$ -MSN (1-13) or  $\alpha$ -MSH (11-13) (10  $\mu$ M). Reactions were stopped after 3 min by aspirating supernatants, immediately adding 1 mL ethanol at -20°C and freezing. cAMP content in the ethanol-soluble fraction was measured using an enzyme immunoassay kit (Amersham).

[0126] Total cellular RNA was extracted from  $10^6$  adherent microglial cells plated in six-well tissue culture plates (Corning). Electrophoresis of RNA samples (10  $\mu$ g/lane) was performed in 1% agarose/2.2 M formaldehyde gels and the gels subsequently blotted onto nylon filters by capillary action and baked for 2 h before prehybridization. The cDNA fragments encoding murine TNF- $\alpha$  and mouse macrophage iNOS were  $^{32}$ P-labeled using the Ready-To-Go DNA Labeling Kit (Pharmacia, Uppsala, Sweden) before hybridization of nylon filters and autoradiography. Blots were subsequently rehybridized with human glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) cDNA probe as an internal control (Clontech Laboratories, Inc., Palo Alto, CA).

[0127] Microglia were plated in 24-well plates at a concentration of  $2 \times 10^5$  cells/ml and  $\alpha$ -MSH production was determined in cell-free supernatants after 24 h incubation with LPS, 10 ng/mL; IFN- $\gamma$ , 1 U/mL; and LPS 10 ng/ml + IFN- $\gamma$ , 1 U/mL,  $\alpha$ -MSH was measured with a double antibody radioimmunoassay (Euro-Diagnostica AB, Malmo, Sweden). The sensitivity of the assay is 0.5 pg/mL and cross-reactivity with other POMC peptides (ACTH (1-24), ACTH (1-39),  $\beta$ -MSH,  $\gamma$ -MSH) is <0.002%.

[0128] Microglia as above in RPMI 1640 medium supplemented with 10% FBS were pre-incubated overnight with rabbit anti- $\alpha$ -MSH produced by resting microglia during the overnight adherence period. After preincubation, the medium was removed and cells co-incubated with LPS 10 ng/mL, IFN- $\gamma$  1 U/mL or LPS + IFN- $\gamma$  diluted in 1 mL RPMI 1640 medium (10% FBS) containing the same rabbit anti- $\alpha$ -MSH antibody concentration used during pre-incubation. The cell-free supernatants were removed after a 24-h incubation and assayed for TNF- $\alpha$ , IL-6, and nitrite. Control samples were treated with rabbit IgG at the same dilution.

[0129] Effects of melanocortin peptides on cytokine and NO production were evaluated by repeated measures analysis of variance followed by Dunnet's test for specific comparisons. Probability values less than 0.05 were considered significant.

[0130] As is shown in Fig. 19 micromolar concentrations of  $\alpha$ -MSH (1-13) significantly reduced IL-6 and  $\text{NO}_2^-$  levels in microglia stimulated with LPS + IFN- $\gamma$  for 24 hr. More particularly,  $\alpha$ -MSH (1-13) reduced the production of TNF- $\alpha$ , IL-6 and  $\text{NO}_2^-$  by 43, 31 and 42% respectively. Fig. 20 shows that  $\alpha$ -MSH (11-13) reduced the production of TNF- $\alpha$ , IL-6 and  $\text{NO}_2^-$  by 45, 50 & 40% respectively. Fig. 21 shows that ACTH (1-24) reduced the production of TNF- $\alpha$ , IL-6 and  $\text{NO}_2^-$  in microglia by 38, 65 and 41% respectively. Figs. 22 and 23 shows that incubation of microglia with  $\alpha$ -MSH (1-13) and  $\alpha$ -MSH (11-13) increased cAMP accumulation in resting cells and cells incubated with LPS + IFN- $\gamma$ . Fig. 24 shows further that the magnitude of cAMP accumulation induced by  $\alpha$ -MSH peptides was comparable to that caused by forskolin.

[0131] As shown in Fig. 25, Northern blot analysis suggests that the inhibitory influence of melanocortin peptides  $\alpha$ -MSH (1-13),  $\alpha$ -MSH (11-13) ACTH 91-24), on TNF- $\alpha$  and  $\text{NO}_2^-$  release was likely caused by the inhibition of mRNA transcription for TNF- $\alpha$  and iNOS.

[0132] These results suggest that the anti-inflammatory activity of the  $\alpha$ -MSH peptides is associated with a reduction in the cytokines connected to the inflammatory response. LPS and IFN- $\gamma$  are signaling molecules that stimulate inflammation by increasing production of cytokines such as TNF- $\alpha$ , IL-6, IL-8, and IL-1. During the resultant inflammatory response, there is a marked increase in production of NO. These results show that microglial cells pre-treated with  $\alpha$ -MSH(1-13),  $\alpha$ -MSH(11-13), or ACTH(1-24) prior to addition of LPS and IFN- $\gamma$  display a

substantial decrease in production of TNF- $\alpha$ , IL-6, and NO.  $\alpha$ -MSH(1-13) was most effective at inhibiting cytokine formation at 10  $\mu$ M,  $\alpha$ -MSH(11-13) was most effective at 1  $\mu$ M, and ACTH(1-24) was most effective at 10-25  $\mu$ M. All three were ineffective at concentrations higher than 50  $\mu$ M. When cells were incubated with anti- $\alpha$ -MSH antibodies before and during treatment with LPS and IFN- $\gamma$ , subsequent treatment with  $\alpha$ -MSH peptides had no effect on cytokine production. This verifies that the reduction in TNF- $\alpha$ , NO, or IL-6 seen in the first experiment was directly attributable to  $\alpha$ -MSH. Northern blot analysis showed that treatment of cells with  $\alpha$ -MSH(1-13) or  $\alpha$ -MSH(11-13) prior to treatment with LPS and IFN- $\gamma$  caused a decrease in transcription levels for both TNF- $\alpha$  and iNOS (inducible NO synthase). These results suggest that the  $\alpha$ -MSH peptides exert their anti-inflammatory effects in part by preventing extracellular signals, such as LPS and IFN- $\gamma$ , from inducing production of pro-inflammatory cytokines, perhaps by blocking signaling pathways that lead to transcription of these cytokines. Experiments were also performed that showed microglial cells produce  $\alpha$ -MSH naturally upon stimulation with LPS and IFN- $\gamma$ , meaning that this peptide sequence is a normal part of the complicated process of inflammation regulation.

[0133] After assessing the effects of the  $\alpha$ -MSH peptides on production of pro-inflammatory cytokines, experiments were run to analyze their effects on cAMP accumulation. One of the first events to occur during the inflammatory response is a decrease in intracellular cAMP levels. The ability of the  $\alpha$ -MSH peptides to induce cAMP accumulation was measured both alone and in conjunction with LPS and IFN- $\gamma$ . Both  $\alpha$ -MSH(1-13) and  $\alpha$ -MSH(11-13) induced cAMP accumulation at concentrations as low as 1  $\mu$ M.  $\alpha$ -MSH(1-13) exhibiting its strongest effect at 10  $\mu$ M, while  $\alpha$ -MSH(11-13) exhibited its strongest effect at 50  $\mu$ M. At all

concentrations tested, the observed accumulation of cAMP was higher than in cells treated with forskolin. When cells were treated with the  $\alpha$ -MSH peptides in conjunction with LPS and IFN- $\gamma$ , there was again a marked increase in cAMP accumulation.  $\alpha$ -MSH(1-13) was the more effective of the two  $\alpha$ -MSH peptides, but  $\alpha$ -MSH(11-13) was also able to induce an accumulation similar to that seen in the presence of forskolin. These results suggest that the  $\alpha$ -MSH peptides exert their anti-inflammatory effect in part by increasing accumulation of cAMP, thus preventing the decrease in cAMP that accompanies the first steps of the inflammatory response. The results of example 9 provide several possible mechanisms by which the  $\alpha$ -MSH peptide sequences appear to prevent inflammation, and again suggest a role for these peptides in the treatment of inflammation in general, and sinusitis in particular.

#### EXPERIMENT 10

[0134] Experiment 10 suggests that preferred compositions according to the invention are comparable to prednisolone for reducing inflammation and accordingly, may serve as therapeutic replacement of prednisolone for the treatment of sinusitis. Experiment 10 compares the anti-inflammatory effects of KPV and the KPV dimer (referred to as (CKPV)<sub>2</sub>) in reducing edema in the hind paw of male ICR CD-1 mice injected with  $\lambda$ -Carrageenan. A total of 162 male ICR CD-1 mice were used in three different experiments. Each mouse received a single subcutaneous injection (20  $\mu$ l) of a carrageenan solution (0.125 % in saline) in each hind paw to induce edema. Paw volume thickness was measured with a micrometer. In order to compare the anti-inflammatory effectiveness of the peptides according to the invention relative to prednisolone, the 162 mice were divided into 10 groups where each group received the following 200  $\mu$ l

dosages (the number of animals is shown in parenthesis: Saline control (31), (CKPV)<sub>2</sub> 0,5 mg/Kg (7), (CKPV)<sub>2</sub> 1,25 mg/Kg (7), (CKPV)<sub>2</sub> 2,5 mg/Kg (16), (CKPV)<sub>2</sub> 5,0 mg/Kg (17), (CKPV)<sub>2</sub> 7,5 mg/Kg (17), KPV 2,5 mg/Kg (9), KPV 5,0 mg/Kg (17), KPV 7,5 mg/Kg (24) and prednisolone suc. 100 mg/Kg (17). The experiment was run for three hours and paw pad thickness was measured at 1.5 hr and 3hr following injection of the anti-inflammatory agents. The results of this experiment are shown in Figure 26. Figure 26 shows that dosages of (CKPV)<sub>2</sub> at 1.25 mg/Kg, 2.5 mg/Kg, 5.0 mg/Kg, and 7.5 mg/Kg and the dosage of KPV at 5.00 mg/Kg, each reduced paw edema by approximately 15-30% versus the approximate 50% reduction in paw edema by prednisolone. Although this experiment suggests that prednisolone is more effective at reducing inflammation than the peptides according to the invention, as taught in the earlier sections, the peptides according to the invention reduce inflammation without the immuno-suppressive and other deleterious side-effects of steroidal anti-inflammatories such as prednisolone.

#### EXAMPLE 11

[0135] A middle age male patient presents complaining of chronic sinus headaches, recurring low grade flu like symptoms and post nasal drip. The patient's history indicates he has had a broad range of allergies to common, airborne allergens since childhood. Blood samples show clinically acceptable white cell counts. The patient is prescribed a pharmacologically effective oral dosage of erythromycin and a pharmacologically effective oral dosage of  $\alpha$ -MSH.

[0136] Although the invention has been described with reference to preferred embodiments and specific examples, it will be readily appreciated by those skilled in the art that many modifications and adaptations of the invention are possible without deviating from the spirit and scope of the invention. Thus, it is to be clearly understood that this description is made  
5 only by way of example and not as a limitation on the scope of the invention as claimed below.

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